European Review for Medical and Pharmacological Sciences 2019; 23: 471-478

High incidence of MTHFR, CBS, and MTRR polymorphisms in vitiligo patients. Preliminary report in a retrospective study

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Abstract. – OBJECTIVE: Vitiligo is a multifactorial polygenic disorder with a complex pathogenesis. It is related to both genetic and no genetic factors. The role of genetics is currently studied with several analytical approaches, such as genetic linkage, candidate gene association studies, genome-wide association studies (GWAS), deep DNA re-sequencing and gene expression studies. To date, there are no genetic traits directly related to vitiligo pathogenesis.

PATIENTS AND METHODS: 43 cases of vitiligo patients and 30 healthy donors recruited as control, were screened by assaying the biochemical molecules involved in the self-cells cytotoxicity (haptoglobin and homocysteine) and candidate genes involved in the regulatory process of the re-methylation cycles and transsulfuration. Candidate genes and their polymorphisms screened are methylene-tetrahydrofolate-reductase (MTHFR) C677T and A1298C; cystathionine-beta-synthase enzyme (CBS) I278T and Ins68bp; and methionine-synthase-reductase (MTRR) A66G.

RESULTS: A peculiar genetic profile in vitiligo patients are defined: 11.6% of vitiligo patients shown polymorphic variant MTHFR 677TT vs. 3.3% of healthy donor MTHFR 677CC profile (p=0.0017); 14.0% of vitiligo patients shown CBS polymorphic variant 278TT vs. 3.3% of healthy donor 278II profile (p=0.0012); and 11.6% of vitiligo patients shown MTRR 66GG vs. 3.3% of healthy donor MTRR 677AA profile (p>0.0001).

CONCLUSIONS: This is the first study reporting the correlation between the polymorphic status of MTHFR C677T, CBS I278T, and MTRR A66G and vitiligo. The genetic screening of these polymorphisms could be useful for early detection of the inheritance risk factor in a subject carrying relatives with vitiligo. Although these data could suggest a kind of dysregulation, genetically based, of thiols production mechanisms. Based on these results, we have not been able to get hypothesis about the putative pathogenesis of vitiligo, and the precise cause remains unclear.

Key Words:
MTHFR C677T, CBS I278T, MTRR A66G, Vitiligo genetic markers.

Introduction

Vitiligo is a common pigmentary disorder of the skin with etiopathogenesis not yet well defined. Clinically vitiligo is characterized by circumscribed, depigmented macules and patches caused by melanocytes apoptosis that is supposed to be in the underlying epidermal layers. Initial lesions occur most frequently on the hands, forearms, feet, and face, favoring a perioral and periocular distribution. The macules are chalk or milk-white in color and are well demarcated. Lesions can be round, oval, or linear in shape[1].

The lack of an international consensus concerning the definition and classification of vitiligo hampers international level research and communication. The Vitiligo European Task Force (VETF) has proposed consensus definitions for the disease, but a need for broader international consensus was felt[2].

Vitiligo is known to affect around 0.1-2% of the world population and could seem at any time from birth to senility[3-5]. The average age of beginning for vitiligo is most commonly observed approximately 20 years[6]. The age of onset is unlikely to vary between the sexes. The vitiligo incidence has been reported female preponderance, but it is

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still not statistically confirmed. This discrepancy has been attributed to an increase in reporting of cosmetic concerns by female patients[7-9]. Etiopathogenesis is almost completely unknown and the clinical dates are conflicting.

Approximately 30% of vitiligo cases occur with familial clustering of cases. The biopsy is occasionally needed for differentiating vitiligo from other hypopigmented or depigmenting disorders. Microscopic examination of involved skin shows a complete absence of melanocytes in association with a total loss of epidermal pigmentation. Superficial perivascular and perifollicular lymphocytic infiltrates may be observed at the margin of vitiliginous lesions, consistent with a cell-mediated process destroying melanocytes[10].

Vitiligo is related to both genetic and no genetic factors. Although several theories have been proposed about the etiopathogenesis of vitiligo, the precise cause remains unknown[11-13]. Commonly agreed upon principles are an absence of functional melanocytes in vitiligo skin and a loss of histochemically recognized melanocytes, owing to their obliteration. Though, the melanocytes damage is most likely a slow process resulting in a progressive decrease of melanocyte depletion activities. Theories regarding the destruction of melanocytes include many factors: autoimmune mechanisms, cytotoxic mechanisms, intrinsic melanocyte defects, oxidant-antioxidant mechanisms, and neural mechanisms[14, 15]. The autoimmune theory proposes an alteration in humoral and cellular immunity in the destruction of melanocytes of vitiligo. This is an actual relevance model, given that no segmental vitiligo is more frequently associated with autoimmune conditions than segmental vitiligo[16-19]. For these reasons, certain disorders have been linked to vitiligo, such as Hashimoto thyroiditis, Graves disease, Addison disease, diabetes mellitus, alopecia aerates, pernicious anemia, inflammatory bowel disease, psoriasis, and autoimmune polyglandular syndrome[20,21]. The most substantial sign of autoimmune pathogenesis is the presence of circulating Immunoglobulin antibodies (Ab) against melanocyte proteins in patients with vitiligo[22-25]. In addition, strong evidence indicates the involvement of humoral cellular immune mechanisms. As a consequence, melanocytes damaging could be directly mediated by T cells autoreactive CD8 positive[26,27].

Vitiligo is characterized by incomplete penetrance, multiple susceptibility loci, and genetic heterogeneity. The inheritance of vitiligo may include genes associated with the biosynthesis of melanin, a response to oxidative stress, and regulation of autoimmunity[28].

Substantial development has been made to understand the role of genetics in vitiligo, with several susceptibility genes being identified[29]. To appreciate the role of genetics in the pathogenesis of vitiligo, several analytical approaches are currently used, such as genetic linkage, candidate gene association studies, genome-wide association studies (GWAS), DNA sequencing studies and gene expression studies[29,30]. To date, there are no genetic traits directly related to vitiligo pathogenesis. Currently, there are no effective therapies for vitiligo. The goal of our research is focused on the genetic and biochemical assessment of mechanisms involved in the self-cells cytotoxicity in patients with vitiligo and their family relatives. This study is performed by assaying the following candidate biochemical and genetic issues:

Analysis of the binding proteins level (haptoglobin, ceruloplasmin) in order to evaluate the binding capability *vs.* molecules potentially harmful to the cell membrane[31].

Analysis of the free plasmatic thiols (homocysteine, glutathione) as markers for the hyper-oxidation and as a mediator of cytotoxicity. In addition, analysis of serum vitamin A and E levels, as part of antioxidant potential, and folates, vitamins B6 and B12 as co-enzyme of the enzymatic activities regulating the methyls' exchange and hemoglobin synthesis[32].

Genetic analysis of the regulatory enzymes genes of the re-methylation cycles and transsulfuration. Candidate genes for polymorphism screening are methylene-tetrahydrofolate-reductase (*MTHFR*) C677T and A1298C[33]; cystathionine-beta-synthase enzyme (*CBS*) I278T and 844Ins68bp[34]; and methionine-synthase-reductase (*MTRR*) A66G[35].

In this issue, we report the genetic profile of the vitiligo patients.

Patients and Methods

Patients

In this study, 43 patients showing vitiligo have been retrospectively enrolled. In addition, 30 healthy donors were used as a control cohort.

This retrospective study was performed in agreement with the Ethical values laid down by according to the Declaration of Helsinki, and informed consent documentation of each patient was

reviewed and agreed by the independent Ethics Committee of AOU "Federico II", Naples, Italy.

Biochemical Assessment of Serum Levels of Circulating Binding Proteins and Vitamins

Analysis of haptoglobin and ceruloplasmin serum level was performed by Nephelometric methods (BN II System – BN Siemens Healthcare Headquarters, Erlangen, Germany);

Analysis of serum Vitamins A and E was performed in High-performance liquid chromatography (HPLC) after extraction of the samples from the organic phase (Hewlett Packard 1100 Series HPLC with Dad Detector, San Diego, CA, USA). Also, in order to evaluate hemoglobin homeostasis, serum Vitamins B6 and B12 are performed by HPLC.

Biochemical Assessment of Thiols Plasma Levels

Analysis of plasma homocysteine and reduced glutathione level was performed by HPLC-reverse phase (HPLC-RF) coupled with fluorescence detection of the free thiols derivative with fluorochrome (Hewlett Packard 1100 Series HPLC with Dad Detector, San Diego, CA, USA).

Genetic Assessment of Polymorphisms

Genotyping of the candidate genes was performed by simple and cost method "Polymerase Chain Reaction-Restriction fragmented length polymorphisms (PCR-RFLP)[36,37], for the following polymorphisms detection:

Polymorphisms C677T and AI298C of the coding gene for the enzyme methylene-tetrahydrofolate-reductase (*MTHFR*)[33]. Restriction Enzyme for allelic discrimination was *Hinf I* for C677T and *MboII* for A1298C.

Polymorphisms I278T and 844-ins68 of the coding gene for the cystathionine-beta-synthase enzyme (*CBS*)[34]. Restriction enzyme for allelic discrimination was *BsrI* for T833C (codon I278T), and conventional PCR for 844ins68.

Polymorphism A66G of the coding gene for the enzyme methionine-synthase-reductase (*MTRR*)[35]. Restriction enzyme for allelic discrimination was *Nde I* for A66G.

Genotyping analysis was performed on genomic DNA by a standard platform using cost-effectiveness criteria approach[36,37]. The entire procedure was performed in accordance with manufacturer's protocols of Dia-Chem Medical Biotechnology (Naples, Italy). PCR Amplification conditions were carried out for 5 minutes at 95°C followed by 35 cycles of 30 s at 94°C, 30 s at 58°C and 1,5 min at 72°C (extension) and a final extension of 7 min at 72°C. PCR product (amplicon) was incubated at 37°C for 4 h for digestion (excluding CBS 844ins68). All products were visualized by 4.0% Agarose gel Nusive® Ethidium bromide-stained.

Genomic DNA was extracted from peripheral blood white cells by a new saline-method following the manufacturer's protocol of *Ampli DNA extraction* (Dia-Chem Srl – Molecular Biology, Naples, Italy).

Statistical Analysis

Mean ± SD (standard deviation) values were calculated in all investigated parameters. Within the Vitiligo patients and healthy controls, frequencies of different polymorphisms investigated were calculated using odds ratio with 95% confidence interval (OR 95%CI) and the χ^2-test, using Software: Stata 13.1 Copyright 1985-2013 StataCorp (College Station, TX, USA). Moreover, we compared the observed frequencies for THRGF genotypes with those predicted in a population by the Hardy-Weinberg equilibrium, using interactive web-tool system calculator[38] http://www.oege.org/software/hwe-mr-calc.shtml.

Results

The examined patients (n=43) shown results statistically significant compared to the control cohort values (n=30).

Serum Levels of Circulating Binding Proteins and Vitamins

As expected the haptoglobin decreased dramatically in vitiligo patients. Levels from 43 patients shown median values 7.6 g/L than controls 20.9 g/L p=0.0043 (Figure 1a). No differences were found in ceruloplasmin level (accounted between 260 and 540 mg/L). The vitamin A, E, B6, and B12 are all in the normal range (data not shown).

Plasmatic Homocysteine Values

This value is averaged higher (12.6 mol/L median of values 10.5-20.7) in patients than controls 9.7 mol/L median of values 8.5-10.7) (Figure 1b). Though, the average homocysteine plasma level is restricted in the normal value of 15 mol/L. This increasing homocysteine plasma level is the result of the genetic profile of the about 30% of pa-

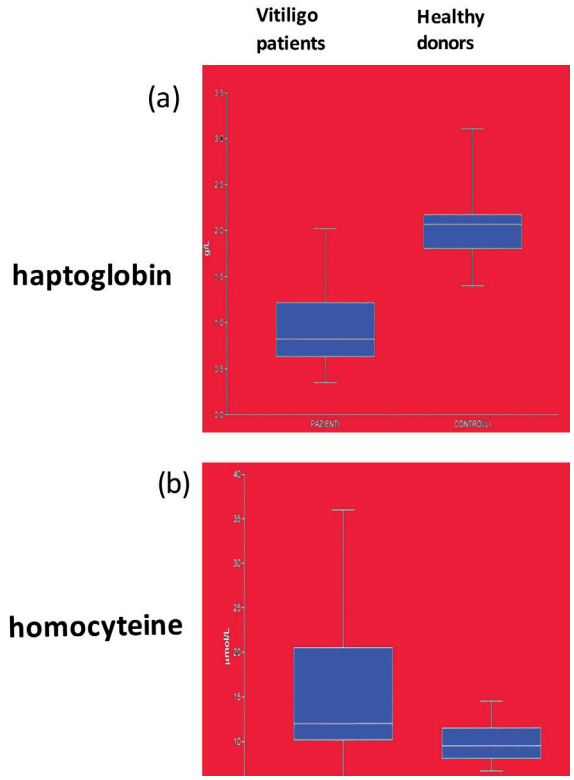

Figure 1. Plot of haptoglobin and homocysteine dosage results. (1a) Serum levels of haptoglobin from 43 patients shown median values 7.6 g/L than controls 20.9 g/L p=0.0043. (1b) Plasmatic homocysteine values is averaged higher (12.6 mol/L median of values 10.5-20.7) in patients than controls 9.7 mol/L median of values 8.5-10.7).

tients who are carrying enzymatic deficit in *CBS, MTHFR* due to polymorphisms (see above).

Increasing in the Prevalence of Polymorphisms in the Enzymes Genes of the Re-Methylation

Genotyping by PCR-RFLP the vitiligo patients and cohort control, reveal unsuspected increasing in the prevalence of polymorphisms in the enzymes genes of the re-methylation (Figure 2, Table I).

We found an increase of the frequency of *MTHFR*-C677T, *CBS*-I278T, and *MTRR*-A66G polymorphisms in homo-and heterozygous, but no evidence in the *MTHFR*-A1298C. As shown in Table II, in the vitiligo cohort we found 5/43 (11.6%) patients carrying homozygous *MTHFR* 677TT and only 1/30 (3.3%) in control cohort p=0.0017. The same result has been found in the MTRR-66GG genotype p>0.0001. The major incidence was found in *CBS*-278TT, which occurred in 6/43 patients (14.0%) and only in 1/30 (3.3%) in controls p=0.0012.

Hardy-Weinberg (HW) equilibrium for, CBS I278T and MTRR A66G and deviated from that expected from a population in equilibrium (CBS I278T χ^2 21.57, p<0.0001; and MTRR χ^2 13.34, p<0.0001), whereas HW equilibrium did not deviate on MTHFR 677 and CBS 844ins68 (all p>0.05).

Discussion

Vitiligo is an acquired "de-pigmentary" skin condition accompanied by severe psychosocial distress, affecting the adult population with a reported preponderance among females of brown skin. The cause and pathogenesis of vitiligo remain unclear. What makes the destruction of melanocytes occur is not understood yet, and uncertainties remain about the natural history and epidemiology of the disease[6].

The first data reported in the literature could suggest a kind of dysregulation, genetically based, of proteins involved in several autoimmune disease and others involved in melanin biosynthesis[26]. Only a few authors believe in the theory of "thiols" production mechanisms and oxidative stress related to melanin biosynthesis[28]. However, we focused our retrospective study in these features, dosing serum levels of the haptoglobin, ceruloplasmin and vitamins A, E, B6, and B12, plasma levels of homocysteine and genotyping for the well-known polymorphisms in genes *MTHFR, CBS*, and *MTRR*[33-35].

The haptoglobin is a plasma circulating protein having the task of binding the serum circulating hemoglobin and directs it to the monocyte-macrophage system for its degradation. The hapto-

Table I.

Gene variants	Resctriction enzyme	Homozygous wild-type	Heterozygous	Homozygous mutant
MTHFR C677T	Hinf I	198 bp	175bp and 23bp	175bp
CBS I278T	Bsr I	174bp	174bp,132bp, 42bp	132bp and 42bp
MTRR A66G	Nde I	231bp and 25bp	231bp, 198bp, 25bp	198bp and 25bp

The size are visualized: 4.0% Agarose gel Nusive Ethidium bromide-stained.

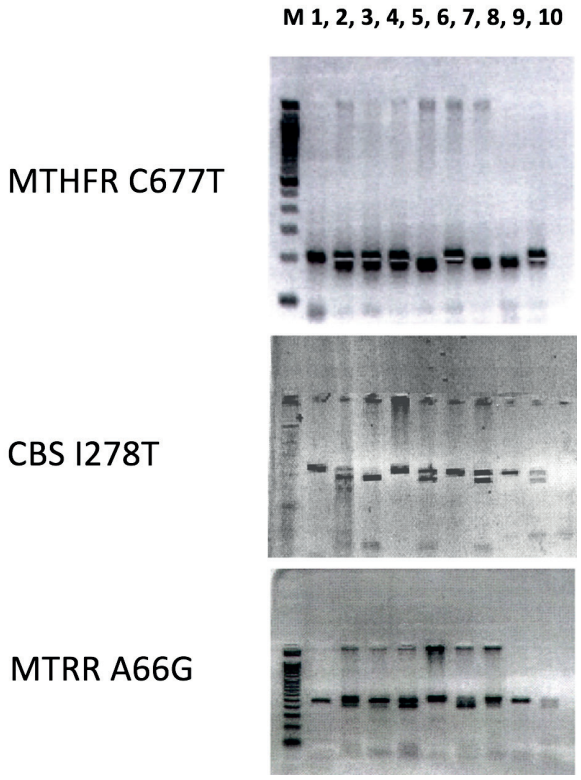

Figure 2. PCR-RFLP results from 9 vitiligo patients. (M) DNA marker Ladder 100 bp. (1-9) example of patients screened for MTHFR, CBS, and MTRR.

globin belongs to the class of proteins called alfa2-globulins and it is a glycoprotein synthesized by the healthy liver[39,40]. The hemoglobin-haptoglobin complex is purified in a few minutes from the blood by the monocyte-macrophage system, or reticuloendothelial system (SRE), allowing the reuse of the substances composing the system, in particular iron. Therefore, due to excessive reuse of haptoglobin will lead to excessive depletion of haptoglobin: as results, vitiligo patients show either the absence or low haptoglobin levels[31].

The synthesis of haptoglobin decreases in patients with hepatic insufficiency and increases in case of inflammation because the hepatic activity will be addressed to inflammation proteins production rather than to proteins normally generated[41].

The binding between the hemoglobin and haptoglobin presents similarities with the one of antigen-antibody. Therefore the decreasing of haptoglobin in vitiligo patients, as shown in our results and literature can be related to a biochemical alteration of the monocyte-macrophage system, or reticuloendothelial system (SRE)[31].

The homocysteine concentration in the blood is related to the genetic factors and environmental factors. The primary mechanism of folic acid is to donate a methyl group in the thiol amino acid synthesis. The folate helps the transfer of a single methyl group in some metabolic responses. The decreasing of vitamins B6 and B9 is due to DNA synthesis and the involved polymorphisms; the following steps are important: the amino acid serine reacts with tetrahydrofolate to form the 5,10-methylenetetrahydrofolate (MTHF). This is the derivative folate in the DNA synthesis. A methyl group is donated to cobalamin by MTHF to create the methylcobalamin.

Thanks to enzyme synthase of methionine, the methylcobalamin donates a methyl group to the homocysteine of metabolite amino acid. This homocysteine is then converted into amino-acid

Table II. Genotyping results.

Gene variants Rs# code	Genetic profile	Control cohort n. 30 (%)	Vitiligo cohort n. 43 (%)	Statistics χ^2-test
MTHFR C677T				
Rs1801133	CC*	17 (56.6)	9 (20.9)	
	CT	12 (40.1)	29 (67.4)	5.41 p=0.0201 (CC vs. CT)
	TT	1 (3.3)	5 (11.6)	9.84 p=0.0017 (CC vs. TT)
CBS T833C (I278T)				
Rs5742905	II*	24 (80.0)	18 (41.9)	
	IT	5 (16.6)	19 (44.2)	6.06 p=0.0138 (II vs. IT)
	TT	1 (3.3)	6 (14.0)	10.52 p=0.0012 (II vs. TT)
CBS 844ins68	No ins*	29 (96.6)	40 (93.0)	
	Ins68	1 (3.4)	3 (7.0)	0.45 p=0.5010 (ins68 vs. No Ins)
MTRR A66G				
Rs10380	AA*	22 (73.3)	9 (20.9)	
	AG	7 (23.3)	24 (55.8)	10.72 p=0.0138 (AA vs. AG)
	GG	1 (3.3)	5 (11.6)	16.66 p>0.0001 (AA vs. GG)

*Wild type gene status.

methionine. The methionine is converted in S-adenosyl-Methionine, a methyl donor operating many biochemical treatments. It is clear that, if the process to produce thyol group is defective, lower biosynthesis of pheomelanin and melanin have been made[28].

Current GWAS has been shown several vitiligo susceptibility genes as histocompatible Leukocyte Antigen A *HLA*-A[42], HLA-DRB1/DQA1[43]. The polymorphism R55Q in the Granzyme B gene (*GZMB*) an apoptotic effector of T-cells, it was found in vitiligo patients with immune disease comorbidities[44]. The polymorphisms S192Y and R402Q, that are common in the Tyrosinase gene (*TYR*), are related as protective genotype for vitiligo incidence in the European population[45]. Furthermore, to date, no genetic traits are directly linked to the etiopathogenesis of vitiligo.

Conclusions

Since the biosynthesis of melanin is directly dependent of the metabolic pathway of amino acid tyrosine (for eumelanin production) and cysteine (for pheomelanin), all phenotype and genotype components could be involved in the pathogenesis of vitiligo[28]. Here, we are reporting important preliminary results relating to polymorphic genotype status of *MTHFR* C677T, *CBS* I278T, and *MTRR* A66G in vitiligo patients. Although, these data could suggest a kind of dysregulation, genetically based, thiols production mechanisms. In the main time, based on these results, we couldn't be able to make a hypothesis about the putative pathogenesis of vitiligo, and the precise cause remains still uncertain.

In addition, in order to screen early the subject with the risk of vitiligo (i.e., relatives), we are proposing a simple genotyping panel assay for detection of the 3 polymorphisms *MTHFR* C677T, *CBS* I278T, and *MTRR* A66G. This panel assay was performed on genomic DNA by a standard platform PCR-RFLP, using cost-benefit criteria approach[36,46].

However, further research is still needed to point its exact mechanism, several hypotheses in recent years have led to a well understanding of the development of this disorder. Many studies have been performed to determine which treatment is the best for vitiligo[47]. Since there is no consensus on the pathogenesis of vitiligo, a treatment to completely cure vitiligo does not exist.

At present, genetics and the autoimmune hypotheses are well supported by several well-founded studies[48].

The genetic screening of these polymorphisms could be useful for early detection of the inheritance risk factor in a subject carrying relatives with vitiligo. Further investigation is necessary to provide better insight into this complicated area, as extended knowledge about immune factors involved in vitiligo lesions formation could lead to the development of new, more efficient therapy, which actually remains only symptomatic.

Conflict of Interest

Disclosures include relationships between GB, CC, MO and MS with DIA-Chem whose products that are related to the subject matter of the manuscript. The other authors stated that there are no conflicts of interest regarding the publication of this article.

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